Supplemental Information

Supplemental Methods

Vaccine Insert Design

PfM115
This 115 kDa composite antigen includes (from N- to C- terminus):

- Block 1 (aa 1-55)
- Block 3 (aa 162-331)
- Block 5 (aa 369-399)
- Block 12 (aa 1079-1170) from 3D7 strain MSP-1 (GenBank Accession NP_704838)
- Wellcome strain block 16/ MSP-1_{33} (aa 1257-1541) (4) (referred to as FVO for consistency in text)
- Glycine-proline linker (GGGPGGG)
- Blocks 16 &17 (MSP-1_{42}) (aa 1320-1709) from strain 3D7 (GenBank Accession NP_704838)

PfM128

This 128 kDa composite antigen includes (from N- to C- terminus):

- Block 1 (aa 1-55)
- Block 3 (aa 162-331)
- Block 5 (aa 369-399)
- Block 12 (aa 1079-1170) from 3D7 strain MSP-1
- Wellcome strain blocks 16 and 17/ MSP-1_{42} (aa 1257-1621) (GenBank Accession X02919)(4) (referred to as FVO for consistency in text)
- Glycine-proline linker (GGGPGGG)
- Blocks 16 &17 (MSP-1_{42}) (aa 1320-1709) from strain 3D7

Three amino acid substitutions were included in one or both block 17 regions: 1609 (S→A, S3A), 1618 (C→ I, C12I) and 1634 (C→W, C28W) (2). The serine substitution was made in order to remove a potential N-glycosylation site. The cysteine residues are located in the first EGF domain and substitutions were made in order to abolish blocking antibody epitopes whilst maintaining inhibitory antibody epitopes and to aid antigen processing (3, 5).

In vitro growth inhibition activity (GIA) assay

IgG was purified from serum using protein G columns and purified polyclonal IgG samples were dialysed and concentrated. Concentrated IgG was pre-absorbed with uninfected human O+ RBCs for 1 hour. *P. falciparum* late trophozoites and schizonts were mixed with test or control samples and culture medium in 96-well culture plates. The final concentration of the culture was 0.3 ± 0.1% parasitaemia, 1% haematocrit in growth medium. Each sample was tested in triplicate. Cultures were maintained for one cycle (40-48 h). Measurement of parasite lactate dehydrogenase (pLDH) was used to quantify relative parasitaemia. Inhibition of growth was determined using the formula :-

$$\%GIA = \left[1 - \left(\frac{A_{650}\text{ sample IgG} - A_{650}\text{ unexposed RBCs}}{A_{650}\text{ unexposed RBCs}}\right)\right] \times 100$$

Intracellular cytokine staining

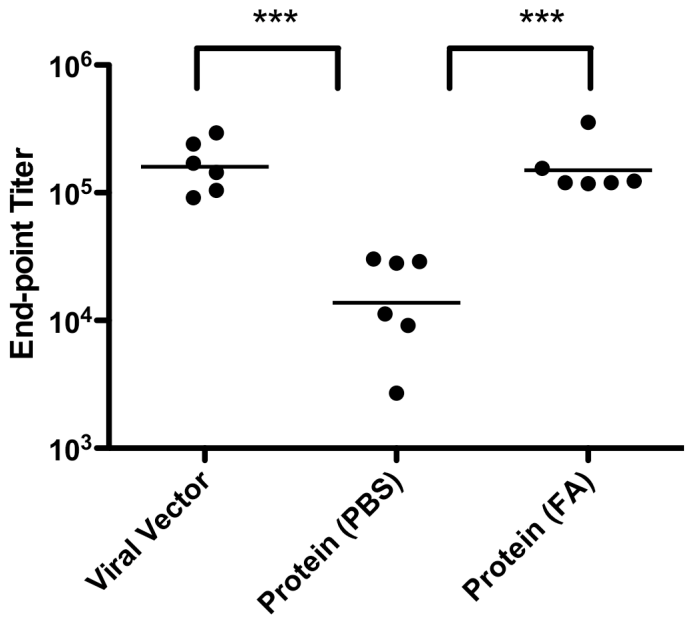
In order to identify T cell epitopes, spleens were taken from 6 mice immunized with AdHu5_M PfM115. Spleens were taken at 2 wks following final immunization. RBC lysis was performed and the remaining cells were resuspended in medium. 15-mer peptides overlapping by 10 aa spanning PfM115 were obtained (Protein Peptide Research) and dissolved in DMSO (20 mg/ml). Pools of 11-20 overlapping peptides corresponding to regions or sub-regions of MSP-1 were prepared. Splenocytes were restimulated for 6 h at 37°C in the presence of Brefeldin A (BD Golgi-Plug) and pooled peptides at a

final concentration of 5 μg/ml per peptide. Stimulated splenocytes were stored at 4°C overnight. Fc block was applied, followed by surface staining for 30 mins at 4°C with CD8 Pacific Blue and CD4 APC Alexa 750 (eBiosciences). Cells were permeabilized using cytofix/cytoperm and intracellularly stained for 1h using IFNγ PE (eBiosciences). Data were analyzed using a Cyan ADP flow cytometer and FloJo. The experiment was repeated using individual peptides and the specific epitopes were identified. T cell epitopes were identified (supplemental table 1). Following identification of T cell epitopes further groups of mice were immunized. Spleens were taken two weeks following the final immunization and splenocytes were restimulated with Brefeldin A and the following peptides; 90/91 (CD8+ responses, BALB/c mice); 86,100,149 & 215 (CD8+ responses, C57BL/6) mice; and 188 (CD4+ responses, C57/BL6 mice) as above. Surface staining was with anti-CD8 PerCP Cy5.5 and anti-CD4 Pacific Blue (eBiosciences). Intracellular staining was with IFNγ APC, TNFα FITC and IL-2 PE. Background responses in wells without peptide were <0.5% (CD8+ IFNγ, TNFα), <0.1% (CD8+ IL-2) or <0.05% (CD4+ IFNγ, TNFα or IL-2).

Statistics

Data were analyzed by parametric tests with the exception of the rabbit experiments and the area under the curve (AUC) analyses. Non-parametric comparisons using Kruskal-Wallis tests were used for rabbit experiments in view of the group size (n = 3 per group). Where a single animal was tested over time paired t-tests were used to compare 'pre-boost' (week 8) titers with 'post-boost' (time-points ≥10 week) titers. Parametric data were analyzed using a t-test or one-way ANOVA with Dunnett's correction. Antibody titers were log-transformed to enable parametric analysis. Correlations were tested using Pearson rank correlations on log-transformed data. Significance was taken as *$P \leq 0.05$, ** $P \leq 0.01$ and ***$P \leq 0.001$. AUC analyses were performed on non-log-transformed data in STATA. AUC was taken as from week 8 (boost) to week 16 (end of experiment). This was calculated for each vaccine regime and the median values were compared using a Kruskal-Wallis test.

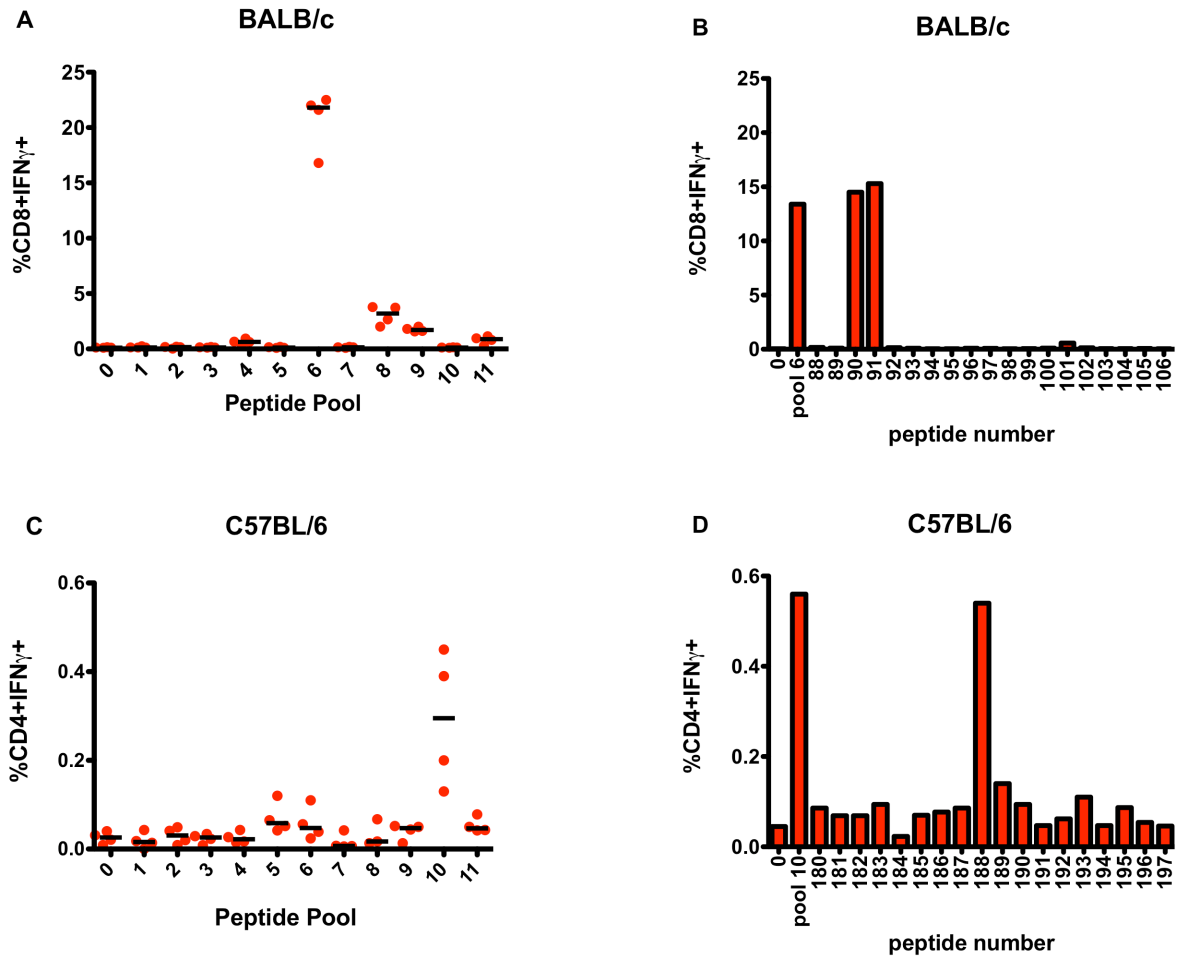
Supplemental Figures



Supplemental Figure 1: *P. falciparum* 3D7 MSP-1_{19}-specific whole IgG antibody responses induced by immunization in BALB/c mice

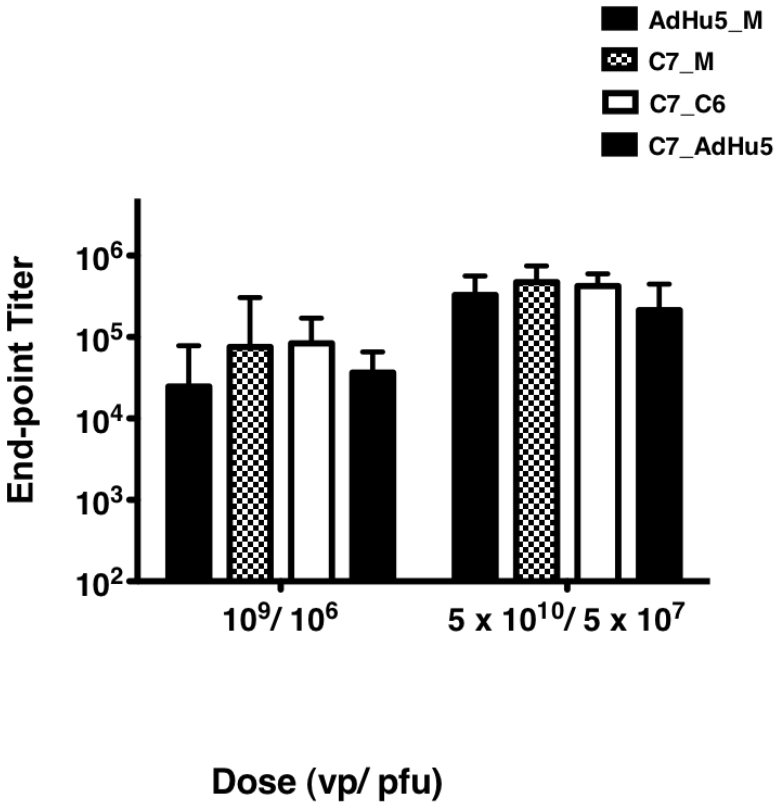
BALB/c mice were immunised s.c. three times with 40µg recombinant PfMSP-1_{19}-C4bp protein IMX108 (Protein) in phosphate buffered saline (PBS) or complete/incomplete Freund's adjuvant (FA). All protein immunizations were two weeks apart. A further group (Viral Vector) of mice were immunised with 5 x 10^{10} v.p. AdHu5 PfM115 followed at week 8 by a boost with 5 x 10^{7} p.f.u. MVA PfM115. Total IgG titers were measured to GST-MSP-1_{19} ETSR in serum samples collected 13 days after the final immunisation. Individual and geomean responses are shown.

*** $P < 0.001$, comparing responses between groups



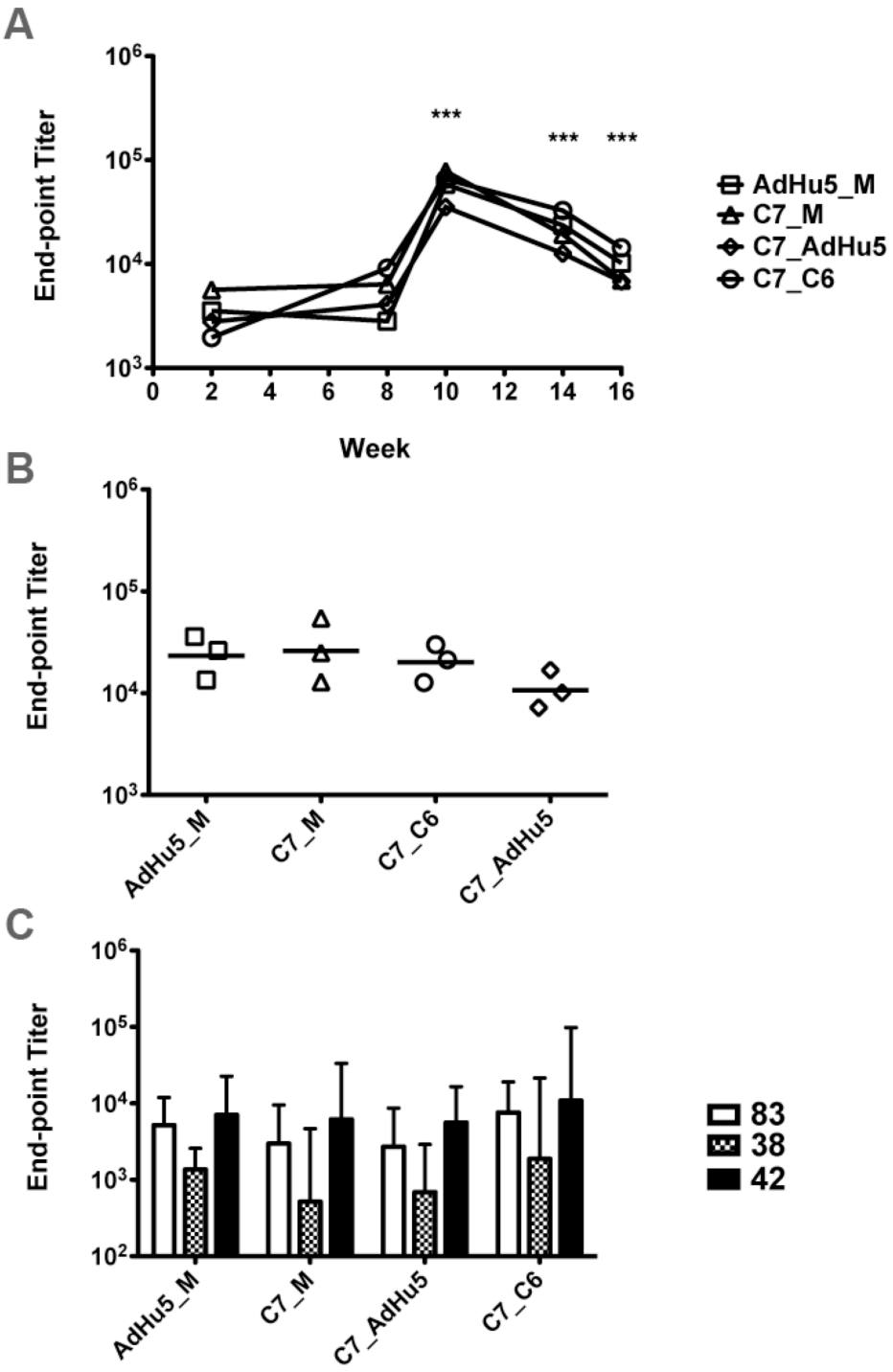
Supplemental Figure 2: MSP-1 Epitopes

Overlapping peptide pools of 15-mers overlapping by 10 aa were used to identify CD8+ and CD4+ T cell epitopes in two strains of inbred mouse. Pool and peptide specific cytokine production was measured from splenocytes of AdHu5_M PfM115 vaccinated mice assessed 2 weeks after the final immunization. Multi-parameter flow cytometry was used to determine the total frequency of IFNγ producing T cells. (*A,B*) CD8+ and (*C,D*) CD4+ T cell responses in BALB/c and C57BL/6 mice respectively. Mean and individual cytokine expressing cells as a percentage of the CD4+ or CD8+ T cell population are shown.



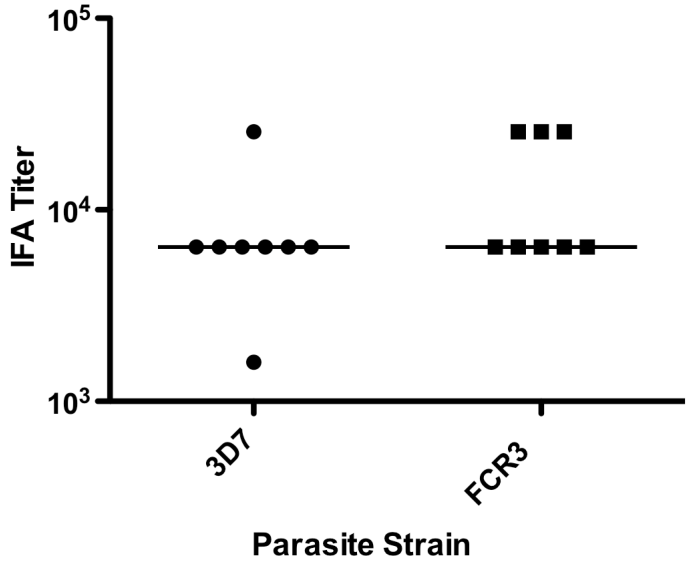
Supplemental Figure 3: Total IgG responses to MSP-1_{19} following low-dose immunization

Mice (n = 6/group) were immunized with adenoviral vectors (AdHu5 or C7) expressing PfM115 and boosted 8 weeks later with MVA PfM115 (M), AdHu5 or C6 as shown (Prime_Boost). Total IgG titers (GMT and 95% CI) were measured by ELISA against GST-MSP-1_{19} (ETSR) in sera taken at week 10. Doses used were 10^9 or 5 x 10^{10} v.p. (adenoviruses) or 10^6 or 5 x 10^7 p.f.u. (MVA) as indicated. All vaccines were given intradermally.



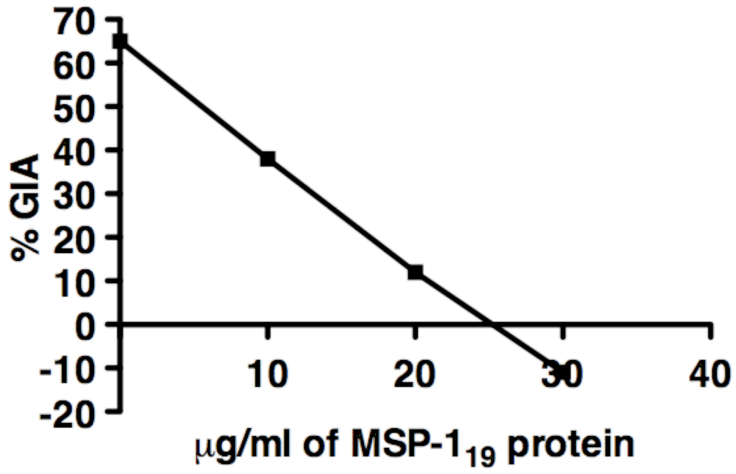
Supplemental Figure 4: Rabbit antibody responses to MSP-1

Rabbits (n = 3/group) were immunized with adenoviral vectors (AdHu5 or C7) expressing PfM115 and boosted 8 weeks later with MVA PfM115 (M), AdHu5 or C6 as shown (Prime_Boost). Total IgG titers were measured by ELISA. (*A*) Geomean titers (GMT) to GST-MSP-1_{19} (ETSR) are shown here plotted over time. (*B*) Individual and GMT to $(His)_6$-tagged MSP-1 at week 10. *(C)* GMT and 95% CI to $(His)_6$-tagged 38, 83 and 42 kDa regions of MSP-1 as shown at week 10. Doses used were 5 x 10^{10} v.p.(adenoviruses) or 10^8 p.f.u. (MVA). All vaccines were given intradermally. *** Different from week 8 GMT ($P < 0.001$)



Supplemental Figure 5: IFA titers

Rabbits (n = 8/group) were immunized with human adenovirus 5 expressing PfM128 and boosted 8 weeks later with MVA PfM128. Doses used were 5 x 10^{10} v.p.(adenoviruses) or 10^8 p.f.u. (MVA). Both vaccines were given intramuscularly. IFA titers were measured using sera taken two weeks following the boost immunization. IFA titers against 3D7 and FCR3 *P. falciparum* are shown with the median response.



Supplemental figure 6: GIA reversal

Purified IgG from five rabbits immunized with the AdHu5_M regime as in **(figure S4)** was pooled and applied to 3D7 *P. falciparum in vitro* at a final concentration of 9 mg/ml. Recombinant MSP-1_{19} (3D7) was also applied at increasing concentrations and was found to reverse the growth inhibitory activity of the rabbit IgG in a dose-dependant manner. Similar inhibition of growth with recombinant MSP-1_{19} was not seen with control sera.

Supplemental table 1: T cell epitopes mapped in PfM115

MURINE STRAIN	PEPTIDE NUMBER	αα POSITION IN MSP-1	MSP-1 BLOCK (STRAIN)	SEQUENCE	CD4/ CD8 EPITOPE	MEAN (%)*
C57BL/6	86	1334-1349	16 (Well)	IPYKDLTSSNYVVKD	CD8	1.2
BALB/c	90 91	1354-1373	16 (Well)	NKEKRDKFLSSYNYI DKFLSSYNYIKDSID	CD8	14.5 15.3
C57BL/6	100 101	1404-1423	16 (Well)	INDKQGENEKYLPFL GENEKYLPFLNNIET	CD8	1.5
C57BL/6	149	1355-1369	16 (3D7)	YRSLKKQIEKNIFT	CD8	2.1
C57BL/6	188	1550-1564	16 (3D7)	DKIDLFKNPYDFEAIK	CD4	0.5
C57BL/6	215	1685-1699	17 (3D7)	TKPDSYPLFDGIFCS	CD8	2.0

* Mean % IFNγ+ cells per total CD8+ or CD4+ T cell subset, results are from two data points from one spleen and were confirmed with a second spleen.

Overlapping peptide pools of 15-mers overlapping by 10 aa were used to identify CD8+ and CD4+ T cell epitopes in two strains of inbred mouse as described in figure S2. Only CD8+ IFNγ+ responses of magnitude > 1.0% were included in this table. Predicted minimal CD8+ epitopes are shown in red font.

Table 2: The effect of amino acid changes in MSP-1_{19} on the binding of a panel of monoclonal antibodies.

Monoclonal Antibody	Effect of mAb on MSP-1_{19} antibody binding to merozoite	Binding to GST-MSP-1_{19} ETSR	Binding to GST-MSP-1_{19} QKNG	Binding to MVA PfM115 (ETSR)	Binding to MVA PfM128 (ETSR & QKNG)
12.8	Inhibitory	+	+	+	+
12.10	Inhibitory	+	+	+	+
111.4	Blocking[1]	-	+	-	+
2.2	Blocking	+	+	-	-
1E1	Blocking[2]	+	+	+	+
7.5	Blocking	+	++	+	+
2F10	Not inhibitory	+	+	+	+

[1] Blocks binding of 1E1 but not 12.8 or 12.10

[2] Atypical blocking activity. Interferes with inhibition

Immunostaining was used to determine whether a panel of monoclonal antibodies bound to MVA PfM115 & PfM128 plaques *in vitro*. Inhibitory antibodies have been previously shown to inhibit processing of MSP-1 and to inhibit erythrocyte invasion (1). Blocking antibodies have been shown to block the binding of inhibitory antibodies, thus enabling MSP-1 to be processed and erythrocyte invasion to occur even in the presence of inhibitory antibodies (5). Binding to GST-MSP-1_{19} was determined by ELISA. Binding to MVA PfM115 and MVA PfM128 was determined by immunostaining. ++ indicates strong binding, + indicates weak binding and – indicates abolition of binding.

Supplemental References

1. **Blackman, M. J., T. J. Scott-Finnigan, S. Shai, and A. A. Holder.** 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J Exp Med **180:**389-93.
2. **Faber, B. W., E. J. Remarque, W. D. Morgan, C. H. Kocken, A. A. Holder, and A. W. Thomas.** 2007. Malaria vaccine-related benefits of a single protein comprising Plasmodium falciparum apical membrane antigen 1 domains I and II fused to a modified form of the 19-kilodalton C-terminal fragment of merozoite surface protein 1. Infect Immun **75:**5947-55.
3. **Hensmann, M., C. Li, C. Moss, V. Lindo, F. Greer, C. Watts, S. A. Ogun, A. A. Holder, and J. Langhorne.** 2004. Disulfide bonds in merozoite surface protein 1 of the malaria parasite impede efficient antigen processing and affect the in vivo antibody response. Eur J Immunol **34:**639-48.
4. **Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, V. Riveros-Moreno, S. C. Nicholls, Y. Hillman, L. S. Davey, M. L. Tizard, R. T. Schwarz, and et al.** 1985. Primary structure of the precursor to the three major surface antigens of Plasmodium falciparum merozoites. Nature **317:**270-3.
5. **Uthaipibull, C., B. Aufiero, S. E. Syed, B. Hansen, J. A. Guevara Patino, E. Angov, I. T. Ling, K. Fegeding, W. D. Morgan, C. Ockenhouse, B. Birdsall, J. Feeney, J. A. Lyon, and A. A. Holder.** 2001. Inhibitory and blocking monoclonal antibody epitopes on merozoite surface protein 1 of the malaria parasite Plasmodium falciparum. J Mol Biol **307:**1381-94.